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Apolipoprotein H Is Not Affected by *In Vitro* Glycosylation

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Increased nonenzymatic glycosylation of all major classes of apolipoproteins has been demonstrated in diabetes. In this work we deal with the *in vitro* nonenzymatic glycosylation of apolipoprotein H, whose role in lipid metabolism is still poorly understood and whose levels increase in diabetes. Apolipoprotein H was isolated from human plasma and purified through a combination of affinity chromatography and continuous elution electrophoresis. The *in vitro* glycosylation was performed by incubating purified apolipoprotein H with high concentration of glucose. Our results indicate that the *in vitro* nonenzymatic glycosylation has no effect on the physical properties of apolipoprotein H, despite the fact that this apolipoprotein contains a high number of lysine residues. Since the *in vitro* concentration of glucose was far higher than the levels normally found in diabetic subjects, it is unlikely for apolipoprotein H to become glycosylated in diabetes.

KEY WORDS: Apolipoprotein H; nonenzymatic glycosylation; affinity chromatography; diabetes mellitus.

1. INTRODUCTION

Nonenzymatic glycosylation of proteins is a process that occurs in diabetes mellitus, and is related to the onset several complications, including cataract formation (Monnier and Cerami, 1981; Monnier *et al.*, 1979), connective tissue abnormalities (Yue *et al.*, 1983; Kent *et al.*, 1985), and macrovascular disease (Witztum *et al.*, 1982; Gonen *et al.*, 1981), the last being one of the major causes of morbidity and mortality in diabetes (Kannel and McGee, 1979).

Moreover, increased nonenzymatic glycosylation of all major classes of apolipoproteins (apo)[4] has been demonstrated in diabetes (Curtiss and Witztum, 1985). The functional consequences of this posttranslational process are related to the abnormalities occurring in lipid metabolism in diabetic subjects (Saudek and Eder, 1979; Gabor *et al.*, 1980). Abnormalities in lipid metabolism and transport are among the main factors contributing to the development of atherosclerosis in diabetic patients. In fact glycosylated apoB renders low-density lipoproteins (LDL) functionally abnormal (Lopes-Virella *et al.*, 1988) as well as immunogenic. They are more susceptible to oxidative modifications than nonglycosylated lipoproteins (Bowie *et al.*, 1993). Recognition of LDL by the LDL receptor can be inhibited by various chemical modifications of the lysine or arginine residues of apoB (Steinbrecher and Witztum, 1984). Many studies have highlighted the role of the lysine residues of apolipoproteins for various functional activities, such as receptor recognition (Weisgraber *et al.*, 1978; Mahley *et al.*, 1979), enzyme interaction and activation (Vainio *et al.*, 1983; Musliner *et al.*, 1979), and lipid binding (Sparrow and Gotto, 1982). Thus, nonenzymatic glycosylation of specific lysine residues of the various apolipoproteins could have a profound influence on the function of that apolipoprotein.

Although the physical and metabolic properties of *in vitro* glycosylated apolipoproteins have been studied at length, similar investigations of apoH have not been

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[4] Abbreviations: apo, apolipoprotein; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic-acid; HPLC, high-pressure liquid chromatography; IEC, ion exchange chromatography; IEF, isoelectrofocusing; LDL, low-density lipoproteins; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, tris-buffer saline.

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performed. In this work we deal with the *in vitro* nonenzymatic glycosylation of apoH, whose role in lipid metabolism is still poorly understood and whose levels increase in diabetes (Cassader *et al.*, 1997).

In the present study apoH was glycosylated *in vitro* in an attempt to explain why apoH levels were found to be elevated in diabetic patients. Glycosylated apoH was subjected to isoelectric focusing (IEF) and ion exchange chromatography (IEC) to prove the nonenzymatic process. Native apoH served as control.

2. MATERIALS AND METHODS

2.1. Materials

Tris, urea, Tween 20, acrylamide, and N,N′-methylene-bis-acrylamide, analytical grade, were purchased from Bio-Rad (Milan, Italy). Gel Bond and ampholytes (pH 4-6.5 and pH 6.5–9) were obtained from Amersham Pharmacia (Milan, Italy); nitrocellulose sheets were from Bio-Rad. Rabbit polyclonal antibody to apoH was kindly supplied by Behring (Scoppito, Italy). Goat anti-rabbit IgG (alkaline phosphatase conjugated), the alkaline phosphatase conjugate substrate kit, and trinitrobenzensulfonic acid were purchased from Sigma (Milan, Italy). Molecular weight standards (low MW) were from Bio-Rad.

2.2. Isolation and Purification of apoH

ApoH was isolated from human plasma and purified through a combination of affinity chromatography and continuous elution electrophoresis as previously described (Gambino *et al.*, 1996). Briefly, rabbit anti-apoH antibodies (22 mg) were immobilized on cyanogen bromide-activated Sepharose 6B. Normal serum samples were diluted into 40 ml of buffer and applied to the column. ApoH bound to the column was eluted with 0.1 M glycine, 0.05% NaN_3, pH 2.5. Sample fractions were concentrated with Centriprep-10 concentrators (Amicon Inc., Beverly, MA) at 3000 rpm and 20°C in a Beckman-J6B centrifuge (Beckman Instruments, Palo Alto, CA) to a final volume of about 1 ml and subjected to continuous elution electrophoresis.

Continuous elution electrophoresis of the proteins (about 2.5 mg) was then performed with a Prep-Cell (Bio-Rad) through a cylindrical gel (gel tube inside diameter 37 mm; gel length 50 mm) composed of 8.5% acrylamide, 2.7% N,N′-methylene-bis-acrylamide, and 0.1% SDS. The buffers were 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.4. Separation was achieved at 40 mA in about 8 hr.

2.3. Glycosylation of apoH

Glycosylation of apoH was performed by incubating purified apoH (0.1 mg/ml) with 0.2 M glucose in sterile PBS containing 1 mM EDTA (pH 7.4) at 37°C for 7 days (Makino *et al.*, 1995).

2.4. SDS–PAGE and Western Blot of Native and Glycosylated apoH

The apolipoprotein purity and the increased electrophoretic mobility due to nonenzymatic glycosylation were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% polyacrylamide gels under nonreducing conditions. Electrophoresis buffer was 25 mM Tris, 0.192 M glycine, 0.1% SDS, pH 8.3.

ApoH was then blotted after SDS–PAGE in 25 mM Tris, 0.192 M Glycine, and 20% methanol, pH 8.3, at 295 mA for 100 min. Nitrocellulose membrane was blocked with 4% bovin serum albumin in 10 mM Tris, 0.15 M NaCl, 0.1% Thimerosal, pH 7.6, and subsequently probed with the specific antibody anti-apoH raised in the rabbit and with the secondary anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase. The blot was developed with the alkaline phosphatase conjugate substrate kit.

2.5. IEF and Western Blot of Native and Glycosylated apoH

IEF was carried out in a Bio-Phoresis TM horizontal electrophoresis cell (Bio-Rad), using a Model 3000/300 power unit. The polyacrylamide gel, *T-5%*, C-3%, contained 2% ampholytes (pH 4–6.5 and 6.5–9; 3.75:1 v/v) and 3 M urea. Cathode and anode solutions were 100 mM NaOH and 40 mM glutamic acid, respectively. After prefocusing at 500 constant V for 35 min, 1 μg of apoH was applied onto each lane. Electrophoresis was performed at 10 constant W for 3 hr. After IEF was performed, the proteins were transferred overnight at room temperature onto 0.45-μm-pore-size nitrocellulose membrane by simple diffusion. After transfer, the nitrocellulose was incubated with specific antibody anti-apoH as described above.

2.6. Assay of Chemical Modification

The number of lysine residues modified in the apolipoprotein upon glycosylation was estimated with the trinitrobenzensulfonic acid assay (Habeed, 1966). The re-

action was carried out in the presence of either potassium thiocyanate or sodium lauryl sulfate in sodium bicarbonate buffer at pH 8.5. The absorbance was read at 335 nm against blank.

2.7. HPLC Ion-Exchange Chromatography

IEC of the apoH was performed by using an anion-exchange column HEMA-IEC Bio 1000 DEAE (7.5×75 mm) (Alltech, Milan, Italy) connected to a Beckman HPLC system. ApoH was applied to the anion column in 20 mM Tris, 0.01% NaN_3, pH 8.0. The protein was eluted with a linear gradient consisting of 20 mM Tris, 0.01% NaN_3 pH 8.0, and 20 mM Tris, 0.01% NaN_3 1 M NaCl, pH 8.0, in 30 min. The absorbance was monitored at 280 nm.

2.8. Dot Blot of Fractions

Rapid screening of HPLC fractions was conducted in the Bio-Dot microfiltration apparatus (Bio-Rad). A sheet of nitrocellulose is clamped between the gasket and the 96-well sample template. Fifty μl of sample is allowed to filter through the membrane. After the antigen is immobilized, the nitrocellulose is incubated in 4% albumin blocking solution. Nitrocellulose is subsequently incubated with rabbit IgG anti-apoH at a 1/2000 dilution at room temperature for 1.5 hr and then with goat IgG anti-rabbit IgG labeled with alkaline phosphatase at a 1/6000 dilution. Dots are visualized with the procedure contained in the Immun-Blot Assay kit (Bio-Rad).

3. RESULTS

Glucose incorporation into apoH was performed by incubating 0.1 mg/ml of apoH with 0.2 M glucose in PBS, pH 7.4, at 37°C for 7 days. After this incubation, apoH was run through an SDS–polyacrylamide slab gel in order to detect any variation in molecular weight.

Figure 1 shows that the molecular weight of glycosylated apoH did not differ from that of apoH incubated under the same conditions in the absence of glucose. Lane 3 contains a fresh sample of apoH; lane 4 contains apoH incubated in the presence of glucose; lane 5 contains apoH incubated in the absence of glucose. These samples were run in the presence of β-mercaptoethanol. Lanes 7–9 contain the same samples run in nonreducing conditions.

Figure 2 illustrates the Western blot of the gel displayed in Fig. 1 and probed with the specific antibodies.

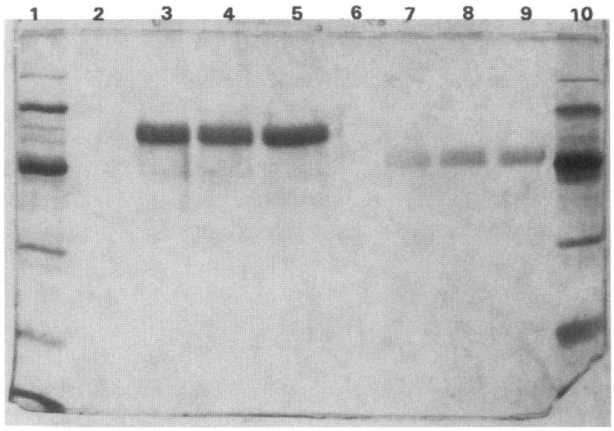

Fig. 1. SDS–PAGE gel electrophoresis of Apo H after incubation of glucose. Lanes 1 and 10: molecular mass markers are (from top to bottom) 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa. Lane 3 contains a fresh sample of apoH; lane 4 contains apoH incubated in the presence of glucose; lane 5 contains apoH incubated in the absence of glucose. These samples were run in the presence of β-mercaptoethanol. Lanes 7–9 contain the same samples run in nonreducing conditions.

ApoH incubated in the presence of glucose had no increase in electrophoretic mobility when compared with apoH incubated in the absence of glucose. From the pictures it is also evident that no proteolytic cleavage had occurred during the incubation at 37°C for 7 days. We thought SDS–PAGE was unable to detect slight differences in molecular weight even when the samples were run under reducing conditions.

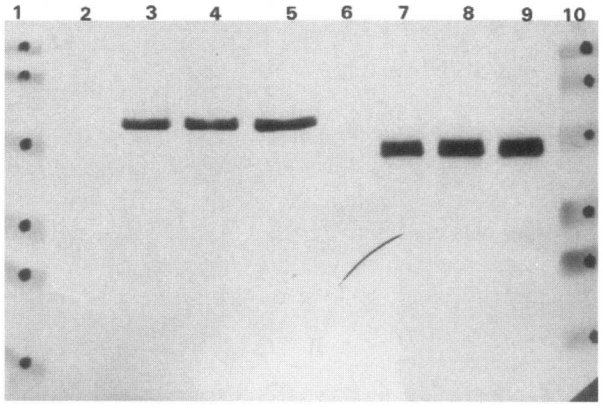

Fig. 2. Western blot of apoH probed with the specific antibody. From left to right: Lanes 1 and 10: prestained molecular mass markers marked with black points are (from top to bottom) 106.0, 80.0, 49.5, 32.5, 27.5, 18.5 kDa. Lane 3 contains a fresh sample of apoH; lane 4 contains apoH incubated in the presence of glucose; lane 5 contains apoH incubated in the absence of glucose. These samples were run in the presence of β-mercaptoethanol. Lanes 7–9 contain the same samples run in nonreducing conditions.

The presence of modified amino groups was assessed with the trinitrobenzensulfonic acid assay. The trinitrobenzensulfonic reagent reacts rapidly with the amino groups of proteins. The reaction was performed in the presence of either potassium thiocyanate or sodium lauryl sulfate in sodium bicarbonate buffer at pH 8.5. In either case there was no difference in absorbance between apoH incubated with glucose and apoH incubated without glucose.

Then, apoH incubated with glucose and apoH incubated in the absence of glucose were analyzed through IEF followed by Western blot. The IEF result is shown in Fig. 3. Lane 1 contains a fresh sample of apoH; lane 2 contains apoH incubated in the presence of glucose; lane 3 contains apoH incubated in the absence of glucose. All the samples show the same number and type of isoforms. The isoelectric point of apoH isoforms was not altered after the incubation with 0.2 M glucose. If the nonenzymatic glycosylation were successful, the positive charge of lysine residues would have been neutralized by glucose. ApoH isoforms should have moved toward more-anodic isoelectric points. The missing shift in isoelectric point reveals that it is unlikely for apoH to become nonenzymatically glycosylated.

The affinity for the anion-exchange column HEMA-IEC did not change after incubating apoH with glucose. Glycosylated apoH should have had greater affinity for the anion-exchange column than native apoH. Both apolipoproteins had symmetrical elution peaks and did not exhibit any chromatographic heterogeneity. Identification of the peak related to apoH was performed through a dot-blot test (data not shown).

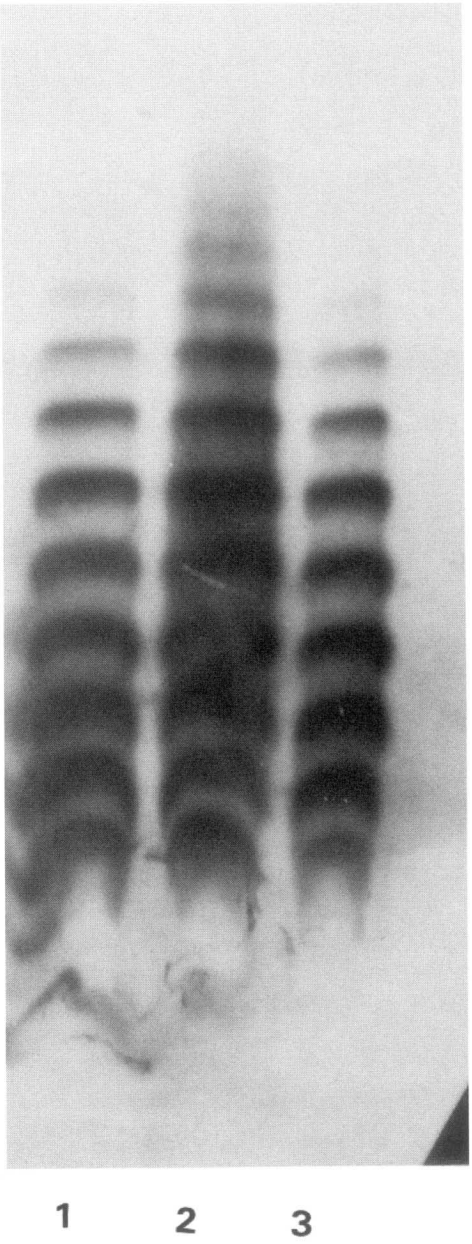

Fig. 3. Western blot of apoH analyzed through IEF. From left to right: Lane 1: native apoH. Lane 2: ApoH incubated in the presence of glucose. Lane 3: ApoH incubated in the absence of glucose.

4. DISCUSSION

Our results indicate that the *in vitro* nonenzymatic glycosylation has no effects on the physical properties of apoH, despite the fact that this apolipoprotein contains a high number of lysine residues. The different chromatography techniques reveal no apparent modification occurring on the apoH molecule. ApoH incubated in the presence or in the absence of glucose had similar electrophoretic mobility when compared after SDS–PAGE under nonreducing conditions. In the presence of reducing agents such as β-mercaptoethanol, the mobility of both samples was higher, but they did not differ from each other. Additionally, both samples had similar affinity for the anion-exchange column, which was due to the integral unmodified lysine residues. The unchanged electrophoretic mobility and the similar affinity are in agreement with the result of IEF, where apoH incubated in the presence of glucose shares the same pattern of isoforms with apoH incubated in the absence of glucose. After glycosylation of lysine residues apoH should lose its positive charge and become more negative. As a result, apoH should move toward more-anodic regions and with a different pattern of isoforms. The failure for apoH to

shift toward anodic regions means that apoH is not prone to becoming nonenzymatically glycosylated under these conditions. Since the *in vitro* concentration of glucose (0.2 M) was far higher than the levels normally found in diabetic subjects, it is unlikely for apoH to become glycosylated in diabetes. This is probably caused by the fact that lysine residues lie in the inner side of apoH molecules and are not accessible for glucose.

Many studies have demonstrated that in diabetic plasma, apoAI, AII, B, CI, E, and albumin are glycosylated (Curtiss and Witztum, 1985; Garlick and Mazer, 1983). It was demonstrated that the modification of lysine 525 altered the conformation of albumin and in turn altered its ability to bind bilirubin and long-chain fatty acids (Shaklai *et al.,* 1984). The glycosylation of proteins could alter protein–lipid interactions. The important role of lysine residues in apoB function and the fact that glycosylation can alter this function has been amply demonstrated (Witztum *et al.,* 1982; Gonen *et al.,* 1981; Makino *et al.,* 1995). On the contrary, apoH can retain its functions even if glucose levels are too high in plasma. Its ability to activate lipoprotein lipase both *in vitro* and *in vivo* could make apoH play a key role in modulation of triglyceride metabolism in diabetes. Further studies are under way to define more precisely the relationship between plasma glucose concentration and the extent of apoH glycosylation and as well as its functional role in diabetes.

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